TOP2A FISH pharmDx™ Kit

Code K5333

1st edition

For in vitro diagnostic use

The kit contains reagents sufficient for 20 tests.

Intended Use

For in vitro diagnostic use

TOP2A FISH pharmDx™ Kit is designed to detect amplifications and deletions (copy number changes) of the *TOP2A* gene using fluorescence in situ hybridization (FISH) technique on formalin-fixed, paraffin-embedded human breast cancer tissue specimens.

Deletions and amplifications of the *TOP2A* gene serve as a marker for poor prognosis in high-risk breast cancer patients.

Results from the *TOP2A* FISH pharmDx™ Kit are intended for use as an adjunct to existing clinical and pathological information.

Summary and Explanation

The clinical performance of Dako *TOP2A* FISH pharmDx™ Kit has been investigated in two studies performed by the Danish Breast Cancer Cooperative Group (DBCG) (1-3). The performance characteristics and clinical utility have been established in a European population.

The data from these studies demonstrate prognostic implications from *TOP2A* amplifications and deletions in breast cancer patients. Overall, patients with tumors showing *TOP2A* amplification have a significantly worse outcome than patients without such amplification. Patients with tumors showing *TOP2A* deletion have even poorer outcome. Prognostic implications with respect to overall survival are present among subgroups of patients treated with chemotherapy regimens that either include or do not include anthracyclines. The presence of predictive implications from *TOP2A* amplifications for optimal use of anthracycline-containing therapy is an area of active research with promising initial results that require grounding in a context of currently available chemotherapeutic options (1, 3-17).

The *TOP2A* gene codes for the enzyme topoisomerase IIα (topo IIα), which catalyzes the breakage and reunion of double-stranded DNA leading to relaxation of DNA supercoils. Type II topoisomerases are essential enzymes that interconvert topological forms of DNA by making transient double-stranded breaks in the DNA backbone (18). These enzymes play important roles in a number of fundamental nuclear processes (19) including DNA replication, transcription, chromosome structure, condensation and segregation (20). The topoisomerase IIα gene, *TOP2A*, is present in 2 copies in all normal diploid cells and is localized to chromosome 17q21 (21). The *TOP2A* gene spans an area of approximately 27.5 kb and contains 35 exons encoding a 170 kDa protein (22).

The topo IIα protein has been recognized as a proliferation marker and the expression of topo IIα varies during cell cycle both in normal and cancerous cells (23). The expression of topo IIα in breast tumors correlates with Ki-67 expression (24-27). No simple relationship has been found for topo IIα at the protein and gene level (24, 26, 27). Only 20% of the topo IIα protein overexpressed cases have *TOP2A* gene amplification but among the *TOP2A* gene amplified cases 93% had overexpression of topo IIα protein (28). Topo IIα overexpression seems to be composed of several contributing factors, both the cancer-specific amplification and the elevated cell proliferation rate. The Ki-67 and topo IIα proteins are expressed in parallel, which can be interpreted as a confirmation of the influence of cell proliferation rate on topo IIα expression, even in cases with *TOP2A* amplification (29).

Type II topoisomerase is a target for anthracyclines such as doxorubicin and epirubicin, which are also termed topoisomerase inhibitors (30-34). Both HER2 status (4, 8, 9) and *TOP2A* status (1, 3-16) have been studied as a marker for treatment with anthracyclines.

The most recent study (1, 3) reports *TOP2A* gene amplification in 12% of breast cancers and deletions with approximately equal frequency when both the HER2 positive and negative tumors are included in the studies. Initially, it was assumed that abnormal *TOP2A* gene copy numbers, as a result of amplification or deletion, were restricted to *HER2* amplified tumors (35, 4). More recently, copy number changes of the *TOP2A* gene have been detected in tumor samples with normal *HER2* gene status (1-3, 5-7, 36, 37).

Principle of Procedure

TOP2A FISH pharmDx™ Kit contains all key reagents required to complete a FISH procedure for routinely processed, formalin-fixed, paraffin-embedded tissue sections.

After deparaffiniztion and rehydration, specimens are heated in Pre-Treatment Solution for 10 minutes. The next step is a proteolytic digestion using ready-to-use Pepsin at room temperature for 5-15 minutes. Following the heating and the proteolytic pre-treatment, this kit employs a ready-to-use FISH Probe Mix based on a combination of PNA (peptide nucleic acid) (38) and DNA technology. This Probe Mix consists of a mixture of Texas Red-labeled DNA cosmid clones covering a total of 228 kb of the *TOP2A* amplicon, and a mixture of fluorescein-labeled PNA probes targeted at the centromeric region of chromosome 17. The specific hybridization to the two targets results in formation of a distinct red fluorescent signal at each *TOP2A* amplicon and a distinct green fluorescent signal at each centromeric region of chromosome 17. To diminish background staining, the Probe Mix also contains unlabeled PNA blocking probes. After a stringent wash, the specimens are mounted with fluorescence mounting medium containing DAPI and coverslipped. Results are interpreted using a fluorescence microscope equipped with appropriate filters (see Appendix 3). Cancer cells are located and then evaluated with regard to the *TOP2A*/CEN-17 signal ratio. Normal cells in the analyzed tissue section will serve as an internal positive control of pre-treatment and hybridization efficiency. For details see Interpretation of Staining section.

TOP2A FISH pharmDx™ Kit, Code K5333, is applicable for manual staining.

Reagents

Materials provided

The materials listed below are sufficient for 20 tests (a test is defined as one 22 mm x 22 mm target area). The number of tests is based on the use of 250 µL per slide of Vial 2 (5-8 drops), 10 µL per slide of Vial 3, and 15 µL per slide of Vial 5. The solutions in Vial 3 and Vial 5 are viscous and may have to be centrifuged shortly in a microcentrifuge in order to be able to collect the entire provided reagent. The kit provides materials sufficient for 10 individual staining runs.

TOP2A FISH pharmDx™ Kit is shipped on dry ice. **To ensure that kit components have not been exposed to high temperatures during transport, dry ice should still be present upon receipt.** Note that some kit components may remain unfrozen, this will not affect the performance of the *TOP2A* FISH pharmDx™ Kit.

Vial 1	PRE-TREATMENT SOLUTION (20x) **Pre-Treatment Solution (20x)** 75 mL, concentrated 20x MES (2-[*N*-morpholino]ethanesulphonic acid) buffer.
Vial 2	PEPSIN **Pepsin** 5 mL, ready-to-use Pepsin solution, pH 2.0; contains stabilizer and an antimicrobial agent.
Vial 3	*TOP2A*/CEN-17 PROBE MIX ***TOP2A*/CEN-17 Probe Mix** 0.2 mL, ready-to-use Mix of Texas Red-labeled *TOP2A* DNA probes and fluorescein-labeled CEN-17 PNA probes; supplied in hybridization buffer with 45% formamide, stabilizer, and unlabeled PNA blocking probes.
Vial 4	STRINGENT WASH BUFFER (20x) **Stringent Wash Buffer (20x)** 150 mL, concentrated 20x SSC (saline-sodium citrate) buffer with detergent.

Vial 5

FLUORESCENCE MOUNTING MEDIUM

Fluorescence Mounting Medium
0.3 mL, ready-to-use
Fluorescence mounting medium with 100 µg/L DAPI (4',6-diamidine-2-phenylindole).

Vial 6

WASH BUFFER (20x)

Wash Buffer (20x)
500 mL, concentrated 20x
Tris/HCl buffer.

COVERSLIP SEALANT

Coverslip Sealant
1 tube, ready-to-use
Solution for removable sealing of coverslips.

NOTE: All reagents, including Pre-Treatment Solution, Stringent Wash Buffer, and Fluorescence Mounting Medium, are formulated specifically for use with this kit. For the test to perform as specified, no substitutions should be made.

Materials required, but not provided

Laboratory reagents

Distilled or deionized water

Ethanol, 96%

Xylene or xylene substitutes

Laboratory equipment

Absorbent wipes

Adjustable pipettes

Calibrated partial immersion thermometer (range 37-100 °C)

Calibrated surface thermometer (range 37-100 °C)

Coverslips (22 mm x 22 mm)

Dako Hybridizer (Code S2450/S2451)*

Forceps

Fume hood

Microcentrifuge (tabletop centrifuge for spinning down probe and mounting media)

Slides, Dako Silanized Slides, Code S3003, or poly-L-lysine-coated slides (see Specimen Preparation)

Staining jars or baths

Timer (capable of 2-15 minute intervals)

Water bath with lid (capable of maintaining 65 (±2) °C to 99 °C)

* Heating block or hybridization oven for denaturation (82 (±2) °C) and hybridization (45 (±2) °C) together with a humid hybridization chamber can be used.

Microscope equipment and accessories

Filters for fluorescence microscope: DAPI and FITC/Texas Red double filter, or FITC and Texas Red mono filters - see Appendix 3 for details

Fluorescence microscope with a 100 watt mercury lamp is recommended
Microscope slide folder (cardboard tray for 20 slides with hinged cover or similar).

Precautions

1. For in vitro diagnostic use.
2. For professional users.
3. The performance characteristics and clinical utility of the *TOP2A* FISH pharmDx™ Kit have been established in a European population.
4. Vial 1, Pre-Treatment Solution (20x), contains 1-<20% 2-morpholinoethanesulphonic acid; Vial 2, Pepsin, contains 5-10% propan-2-ol; Vial 4, Stringent Wash Buffer (20x), contains 1-<5% octoxinol; and Vial 6, Wash Buffer (20x), contains 1-<20% trometamol. At product concentrations these substances do not require hazard labeling. Material Safety Data Sheets (MSDSs) are available for professional users on request.
5. Vial 2, Pepsin, contains pepsin A that may cause an allergic reaction.
6. Vial 3, *TOP2A*/CEN-17 Probe Mix contains 45% formamide and is labeled:
   Toxic.
   R61 May cause harm to the unborn child.
   S45 In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).
   S53 Avoid exposure – obtain special instructions before use. Hybridization should take place in a fume hood.
   S60 This material and/or its container must be disposed of as hazardous waste.
   As a general rule, persons under 18 years of age are not allowed to work with this product. Users must be carefully instructed in the proper working procedure, the dangerous properties of the product and the necessary safety instructions. Please refer to the Material Safety Data Sheet (MSDS) for additional information (per European Union Directive 94/33/EC).
7. Coverslip Sealant contains 60-100% naphtha (petroleum), hydrotreated light, and is labeled:
   Extremely flammable.
   Dangerous for the environment.
   R11 Highly flammable.
   R51/53 Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.
   S9 Keep container in a well-ventilated place.
   S16 Keep away from sources of ignition – No smoking.
   S35 This material and its container must be disposed of in a safe way.
   S57 Use appropriate container to avoid environmental contamination.
   S61 Avoid release to the environment. Refer to special instructions/safety data sheets.
   Please refer to the Material Safety Data Sheet (MSDS) for additional information.
8. Specimens, before and after fixation, and all materials exposed to them, should be handled as if capable of transmitting infection and should be disposed of with proper precautions (39). Never pipette reagents by mouth and avoid contacting the skin and mucous membranes with reagents and specimens. If reagents come in contact with sensitive areas, wash with copious amounts of water.
9. Minimize microbial contamination of reagents to avoid erroneous results.
10. Incubation times and temperatures, or methods other than those specified, may give erroneous results.
11. Tissue fixation methods and thickness of specimen other than those specified may affect tissue morphology and/or signal intensity.
12. Avoid evaporation of *TOP2A*/CEN-17 Probe Mix during hybridization by ensuring sufficient humidity in the hybridization chamber.
13. Reagents have been optimally diluted. Further dilution may result in loss of performance.
14. Wear appropriate personal protective equipment to avoid contact with eyes and skin. Please refer to the Material Safety Data Sheet (MSDS) for additional information.

Storage

Store reagents at 2-8 °C in the dark. All reagents tolerate frozen storage. Freezing and thawing the kit for each analysis does not affect performance.

The Ready-to-Use Pepsin, *TOP2A*/CEN-17 Probe Mix, and Fluorescence Mounting Medium (Vials 2, 3 and 5) may be affected adversely if exposed to heat. Do not leave these components at room temperature. The *TOP2A*/CEN-17 Probe Mix and Fluorescence Mounting Medium (Vials 3 and 5) may be affected adversely if exposed to excessive light levels. Do not store these components or perform analysis in strong light, such as direct sunlight.

Do not use the kit after the expiration date stamped on the outside of the package. If reagents are stored under conditions different from those specified in this package insert, the user must validate reagent performance (40).

There are no obvious signs indicating instability of this product. Therefore, it is important to evaluate normal cells in the analyzed tissue section. If an unexpected fluorescence pattern is observed, which cannot be explained by variations in laboratory procedures, and a problem with *TOP2A* FISH pharmDx™ Kit is suspected, contact Dako Technical Services immediately.

Specimen Preparation

Specimens from biopsies must be handled to preserve the tissue for FISH analysis. Standard methods of tissue processing for immunohistochemical staining should be used for all specimens (41).

Paraffin-embedded sections

Only tissue preserved in neutral-buffered formalin and paraffin-embedded is suitable for use. Specimens from biopsies should e.g. be blocked into a thickness of 3 or 4 mm and fixed for 18-24 hours in neutral-buffered formalin. The tissues should then be dehydrated in a graded series of ethanol and xylene, followed by infiltration by melted paraffin held at no more than 60 °C.

Properly fixed and embedded tissues will keep indefinitely prior to sectioning and slide mounting if stored in a cool place (15-25 °C) (41, 42). Other fixatives are not suitable.

Tissue specimens should be cut into sections of 4-6 µm.

The slides required for *TOP2A* gene aberration analysis and verification of tumor presence should be prepared at the same time. A minimum of 3 serial sections is recommended, 1 section for tumor presence stained with hematoxylin and eosin (H&E stain), 1 section for *TOP2A* gene aberration analysis, and 1 section for back up. It is recommended that tissue sections are mounted on Dako Silanized Slides, Code S3003, SuperFrost Plus, or poly-L-lysine slides. Specimens should be analyzed within 4-6 months of sectioning when stored at room temperature (20-25 °C) or 2 years when stored at 2-8 °C.

Stability of cut sections at different storage conditions:

- Room temperature (20-25 °C): 4-6 months
- Refrigerator (2-8 °C): 2 years

INSTRUCTIONS FOR USE

A. Reagent Preparation

It is convenient to prepare the following reagents prior to staining:

A.1 Pre-Treatment Solution

Crystals may occur in Vial 1, but will dissolve at room temperature. Ensure that no crystals are present before preparation of reagent.

Dilute a sufficient quantity of Vial 1 (Pre-Treatment Solution 20x) 1:20 using distilled or deionised water. Unused diluted buffer may be stored at 2-8 °C for one month. Discard buffer if cloudy in appearance.

A.2 Stringent Wash Buffer

Dilute a sufficient quantity of Vial 4 (Stringent Wash Buffer 20x) 1:20 using distilled or deionised water. Unused diluted buffer may be stored at 2-8 °C for one month. Discard buffer if cloudy in appearance.

A.3 Wash Buffer

Dilute a sufficient quantity of Vial 6 (Wash Buffer 20x) 1:20 using distilled or deionised water. Unused diluted buffer may be stored at 2-8 °C for one month. Discard diluted buffer if cloudy in appearance.

A.4 Ethanol series

From a 96% ethanol solution, prepare 3 jars with 70%, 85%, and 96% ethanol respectively. Store covered jars at room temperature or at 2-8 °C, and use for a maximum of 200 slides. Discard if solutions appear cloudy.

B. Staining Procedure

B.1 Procedural notes

The user should read these instructions carefully and become familiar with all components prior to use (see Precautions).

If kit components are stored frozen, it is recommended to move the reagents to 2-8 °C the day before performing the analysis to allow proper temperature equilibration. All reagents should be equilibrated to the relevant temperature prior to use as follows.

Vial 1, The diluted Pre-Treatment Solution should be equilibrated to **95-99 °C**.
Vial 2, Pepsin should be applied at **2-8 °C** and kept cold continuously.
Vial 3, *TOP2A*/CEN-17 Probe Mix may be applied at any temperature from **2-25 °C**.
Vial 4, Diluted Stringent Wash Buffer, one jar should be equilibrated to room temperature, the other to **65 (±2) °C** prior to use.
Vial 5, Fluorescence Mounting Medium may be applied at any temperature from **2-25 °C**.
Vial 6, The diluted Wash Buffer should be equilibrated to room temperate **20-25 °C**.
Coverslip Sealant may be applied at any temperature from **2-25 °C**.

All steps must be performed at the outlined temperature.

The procedure includes a number of dehydrations followed by drying of the tissue sections. Ensure that tissue sections are completely dry before proceeding to the next step. Do not allow tissue sections to dry during the other procedural steps.

If the staining procedure has to be interrupted, slides may be kept in Wash Buffer after the deparaffinization step for up to 1 hour at room temperature (20-25 °C) without affecting the results.

B.2 Treatment of tissues prior to staining

Deparaffinization and rehydration: Prior to performing the analysis, tissue slides must be deparaffinized to remove embedding medium and rehydrated. Avoid incomplete removal of paraffin.

Residual embedding medium will result in increased non-specific staining. This step should be performed at room temperature (20-25 °C).

1. Place slides in a xylene bath and incubate for 5 (±1) minutes. Change baths and repeat once.
2. Tap off excess liquid and place slides in 96% ethanol for 2 (±1) minutes. Change baths and repeat once.
3. Tap off excess liquid and place slides in 70% ethanol for 2 (±1) minutes. Change baths and repeat once.
4. Tap off excess liquid and place slides in diluted Wash Buffer (see INSTRUCTIONS FOR USE, Section A.3) for a minimum of 2 minutes. Commence staining procedure as outlined in Section B.3, Step 1, Pre-Treatment.

Xylene and alcohol solutions should be changed after 200 slides or less.

Xylene substitutes may be used.

NOTE: The reagents and instructions supplied in this kit have been designed for optimal performance. Further dilution of the reagents or alteration of incubation temperatures may give erroneous or discordant results. Differences in tissue processing and technical procedures in the user's laboratory may invalidate the assay results.

B.3 Staining protocol

DAY 1

Step 1: Pre-Treatment
Fill a staining jar with the diluted Pre-Treatment Solution (see INSTRUCTIONS FOR USE, Section A.1). Place the staining jar containing Pre-Treatment Solution in a water bath. Heat water bath and the Pre-Treatment Solution to 95-99 °C. Measure temperature inside the jar with a calibrated thermometer to ensure correct temperature. Cover the jar with a lid in order to stabilize the temperature and avoid evaporation.

Immerse the room temperature deparaffinized sections into the preheated Pre-Treatment Solution in the staining jar. Re-check temperature and incubate for 10 (±1) minutes at 95-99 °C.

Remove the entire jar with slides from the water bath. Remove lid and allow the slides to cool in the Pre-Treatment Solution for 15 minutes at room temperature.

Transfer the slides to a jar with diluted Wash Buffer (see INSTRUCTIONS FOR USE, Section A.3) for 3 minutes at room temperature (20-25 °C).

Replace Wash Buffer and soak sections for another 3 minutes.

NOTE: The Pre-Treatment Solution is designed for a single use application only. Do not re-use.

Step 2: Pepsin, ready-to-use
Tap off excess buffer. Using lintless tissue (such as an absorbent wipe or gauze pad), carefully wipe around the specimen to remove any remaining liquid and to keep reagents within the prescribed area.

Apply 5-8 drops (250 µL) of cold (2-8 °C) Pepsin (Vial 2) to cover specimen. Always store Pepsin at 2-8 °C.

Incubate for 5-15 minutes at room temperature (20-25 °C). An incubation time of 10 minutes will be adequate for most specimens, but the optimal incubation time may depend on tissue fixation and/or thickness of specimen and should be determined by the user.

Tap off Ready-to-Use Pepsin and soak sections in the diluted Wash Buffer (see INSTRUCTIONS FOR USE, Section A.3) for 3 minutes at room temperature (20-25 °C).

Replace Wash Buffer and soak sections for another 3 minutes.

Dehydrate tissue sections through a graded series of ethanol: 2 minutes in 70% ethanol, 2 minutes in 85% ethanol, and 2 minutes in 96% ethanol.

Allow tissue sections to air dry completely.

Step 3: *TOP2A*/CEN-17 Probe Mix
The following step should be performed in a fume hood.

Apply 10 µL of *TOP2A*/CEN-17 Probe Mix (Vial 3) to the centre of the tissue section. Immediately place a 22 mm x 22 mm glass coverslip over the Probe Mix and allow it to spread evenly under the coverslip. Avoid air bubbles. If air bubbles are observed, gently tap them away from the tissue using forceps.

Seal coverslip with Coverslip Sealant by ejecting the Sealant around the periphery of the coverslip. Allow the Coverslip Sealant to overlap the coverslip and the slide, thereby forming a seal around the coverslip. Make sure that the Coverslip Sealant covers the entire edge of the coverslip.

Prepare Dako Hybridizer* (Code S2450/S2451) for a hybridization run. Make sure that Humidity Control Strips (Code S2452) are wet and optimal for use. Start the Hybridizer and choose a program that will denature at 82 °C for 5 minutes and hybridize overnight (14-20 hours) at 45 °C (please refer to Dako Hybridizer Instruction Manual for details).

Place slides in the Hybridizer, make sure the lid is properly closed and start program.

*Instrumentation that allows for conditions similar to the ones described above may be used for denaturation and hybridization, e.g. as described below:

Place slides on a flat metal or stone surface (heating block or on a block in a hybridization oven) preheated to 82 (±1) °C. Denature probe and target DNA for 5 minutes ensuring that the temperature of the block does not drop below 80 °C at any time.

Place slides in a preheated humidified hybridization chamber. Cover the chamber with a lid and incubate overnight (14-20 hours) at **45 (±2) °C**. Please note that a hybridization temperature of 37 °C is not suitable for use with the probes contained within this kit.

DAY 2

Step 4: Stringent Wash
Fill two staining jars with the diluted Stringent Wash Buffer (see INSTRUCTIONS FOR USE, Section A.2). A minimum volume of 100 mL or 15 mL per slide in each jar is recommended.

Place one of the staining jars containing diluted Stringent Wash Buffer at room temperature in a fume hood and the other in a water bath. Heat water bath and the diluted Stringent Wash Buffer to 65 (±2) °C. Ensure that the temperature has stabilized. Cover jar with lid in order to stabilize the temperature and avoid evaporation. Measure temperature inside the water bath jar with a calibrated thermometer to ensure correct temperature. The Stringent Wash Buffer contains detergent and may become turbid at 65 °C; this will not affect performance.

Using forceps or gloves, take slides from the hybridization chamber and gently remove Coverslip Sealant as well as coverslip and place slides in the room temperature pre-wash jar, one at a time. Do not place slides in Stringent Wash Buffer before removing coverslips.

As soon as all coverslips have been removed, transfer slides from the room temperature pre-wash jar to the 65(±2) °C jar in the water bath. Perform stringent wash for exactly 10 minutes at 65 (±2) °C.

Remove slides from the diluted Stringent Wash Buffer, and soak sections in diluted Wash Buffer for 3 minutes at room temperature (20-25 °C).

Change diluted Wash Buffer and soak sections for another 3 minutes.

Dehydrate tissue sections through a graded series of ethanol: 2 minutes in 70% ethanol, 2 minutes in 85% ethanol, and 2 minutes in 96% ethanol.

Allow tissue sections to dry completely.

Step 5: Mounting
Apply 15 µL of Fluorescence Mounting Medium containing DAPI (Vial 5) to the target area of the slide and apply a glass coverslip.

NOTE: Slides may be read after 15 minutes or within 7 days after mounting. However, fading occurs if slides are exposed to light or high temperatures. To minimize fading, store slides in the dark at 2-8 °C.

Quality Control

1. Signals must be bright, distinct and easy to evaluate.
2. Normal cells allow for an internal control of the staining run.
   - Normal cells should have 1-2 clearly visible green signals indicating that the CEN-17 PNA Probe has successfully hybridized to the centromeric region of chromosome 17.
   - Normal cells should also have 1-2 clearly visible red signals indicating that the *TOP2A* DNA Probe has successfully hybridized to the *TOP2A* target region.
   - Due to tissue sectioning, some normal cells will have less than the expected 2 signals of each color.
   - Normal cells undergoing cell division may have more than the normal 1-2 signals of each color.
   - Failure to detect signals in normal cells indicates assay failure, and results should be considered invalid.
3. Nuclear morphology must be intact when evaluated using a DAPI filter. Numerous ghost-like cells and a general poor nuclear morphology indicate over-digestion of the specimen, resulting in loss or fragmentation of signals. Such specimens should be considered invalid.
4. Differences in tissue fixation, processing, and embedding in the user's laboratory may produce variability in results, necessitating regular evaluation of in-house controls.

Interpretation of Staining

Assessable Tissue

Only specimens from patients with invasive carcinoma should be tested. In cases with carcinoma in situ and invasive carcinoma in the same specimen, only the invasive component should be scored. Avoid areas of necrosis and areas where the nuclear borders are ambiguous. Do not include nuclei that require subjective judgment. Skip nuclei with weak signal intensity and non-specific or high background. Use the DAPI-filter to check for even staining of the nuclei.

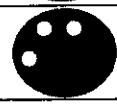
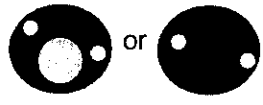
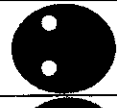
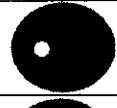
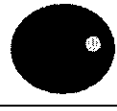
Signal enumeration

Locate the tumor within the context of the H&E stained slide and evaluate the same area on the FISH stained slide. Scan several areas of tumor cells to account for possible heterogeneity. Select an area having good nuclei distribution. Begin analysis in the upper left quadrant of the selected area and, scanning from left to right, count the number of signals within the nuclear boundary of each evaluated nucleus according to the guidelines below (see also Appendix 3).

- Focus up and down to find all of the signals in the individual nucleus.
- Count two signals that are the same size and separated by a distance equal to or less than the diameter of the signal as only one signal.
- In nuclei with high levels of *TOP2A* gene amplification, the *TOP2A* signals may be positioned very close to each other forming a cluster of signals. In these cases the number of *TOP2A* signals cannot be counted, but must be estimated. Special attention must be paid to the green signals, as clusters of *TOP2A* signals can cover the green signals making them impossible to see. In case of doubt, please check the green signals using a specific FITC filter.
- Do not score nuclei with no green signals. Score only those nuclei with one or more green reference signals. The ratio is calculated as red signals/green signals and the denominator cannot be 0.
- Record counts in a table as shown in Appendix 2.

Signal counting guide

1		Do not count. Nuclei are overlapping, not all areas of nuclei

		are visible
2		Count as two green signals
3		Two red signals, do not score nuclei with only red signals (denominator in the ratio cannot be 0)
4		Count as 3 green and 12 red signals (cluster estimation)
5		Count as 1 green and 1 red signal. Two signals of the same size and separated by a distance equal to or less than the diameter of one signal are counted as one
6		Do not count (over- or underdigested nuclei). Missing signals in the centre of nuclei (donut-shaped nuclei).
7		Count as 2 green and 3 red signals. Two signals of the same size and separated by a distance equal to or less than the diameter of one signal are counted as one
8		Count as 1 green and 5 red signals
9		Count as 3 green (1 green out of focus) and 3 red signals
10		Cluster of red signals hiding green signals, check the green signals with a specific FITC filter, or do not count

The signals can be scored either by the conventional method (43) or by an alternative, time- and labor-reducing method (1, 2). Instead of the conventional method of counting signals in 60 nuclei, a total of 60 events are scored, where one event is a red gene signal. By this alternative counting method a variable number of nuclei are scored until 60 red *TOP2A* signals are reached. The corresponding green CEN-17 signals in the same nuclei are recorded. The minimum number of nuclei to score is 6. In normal specimens an average of 35 nuclei will be enough to reach 60 red signals. In amplified cases 6-35 nuclei will be included. Even in cases with deletions, less than 60 nuclei will often be sufficient. The latter method has the advantage that the highest number of cells will be counted in the deleted and normal cases, while the lowest number of cells will be counted in the amplified cases. These cases are often obvious to identify just by looking in the microscope, but are quite tedious and time-demanding to count if 60 nuclei should be scored. The concordance between the two counting methods was high. In the reproducibility study (see Table 8) comparable ratios were obtained and 89/90 slides showed concordant results.

If possible count the nuclei from 3 distinct tumor areas (44). Calculate the *TOP2A*/CEN-17 ratio by dividing the total number of red *TOP2A* signals by the total number of green CEN-17 signals.

Specimens with a *TOP2A*/CEN-17 ratio above or equal to 2.00 should be considered as having *TOP2A* amplification and specimens with a *TOP2A*/CEN-17 ratio less than 0.80 should be considered as having *TOP2A* deletion (1, 2, 9).*TOP2A* status was categorized into the following groups:

- Deleted: *TOP2A*/CEN-17 ratio < 0.80
- Normal: 0.80 ≤ *TOP2A*/CEN-17 ratio < 2.00
- Amplified: *TOP2A*/CEN-17 ratio ≥ 2.00

Results at or near the cut-off (1.80-2.20 for amplifications and 0.70-0.90 for deletions) should be interpreted with caution. It is recommended to check that the scorings do not include a high percentage of normal nuclei.

If the ratio falls into either of the two equivocal zones, or in case of uncertainty, it is recommended that the score of the specimen should be verified by rescoring by a second person, by counting nuclei from 3 more tumor areas and/or counting a total of 60 nuclei. The final ratio should be recalculated based on all scorings. For borderline cases a consultation between the pathologist and the treating physician is warranted.

Expected values

Testing of normal tissue

To establish a range of results for normal tissue, a study was conducted that measured the distribution of *TOP2A*/CEN-17 ratios in normal breast tissue specimens using the recommended scoring method of 60 nuclei. In a sample set of 21 normal breast tissue specimens, the median *TOP2A*/CEN-17 ratio was 1.08 with the 2.5 and 97.5 percentiles forming an interval of 1.00 to 1.20.

Selection of cut-off

The reasons for using FISH ratio ≥ 2.0 as the cut-off for gene amplification are in line with those presented by Press and coworkers (12) 1) The established "cut-off" used for evaluating gene amplification with Southern Blot was originally a ratio of an index gene-to-control of 2.0 or greater. 2) The accepted FDA-approved FISH ratio for *HER2* gene amplification is ≥ 2.0 and there are no data proving that another ratio should be chosen for *TOP2A*. 3) Because only a portion of a cell population is dividing at any one time, using a ratio of 2.0 or greater is not likely to lead to confusion with non-amplified active dividing cell populations. A FISH ratio of ≤ 0.8 as indicative of *TOP2A* gene deletion has been selected because it allows identification of breast cancers that lose a single gene copy from a tetraploid or near-tetraploid, anauploid breast cancer. In addition, this is the ratio that has been used by the majority of investigators as listed in Table 1 below.

Table 1. *TOP2A*/CEN-17 ratios used in different studies reported in the literature

Study	Cut-off *HER2* amplification	Cut-off *TOP2A* amplification	Cut-off *TOP2A* deletion	Reference
Järvinen, 1999	1.5	1.5	0.67	(35)
Järvinen, 2000	1.5	1.5	0.7	(4)
Di Leo, 2002	2.0	1.5	0.8	(9)
Coon, 2002	2.5	2.5	Ni	(8)
Park, 2003	4 (CISH)[1]	4 (CISH)[1]	Ni	(10)
Bofin, 2003	2.0	2.0	1.0	(37)
Olsen, 2004	2.0	2.0	0.8	(2)
Cardoso, 2004	2.0	1.5	ND	(29)
Durbecq, 2004	2.0	1.5	Ni	(26)
Hicks, 2005	2.0	2.0	0.7	(45)
Knoop, 2005	2.0	2.0	0.8	(1, 3)
Callagy, 2005	1.5	1.5	Ni	(28)
Press, 2005	2.0	2.0	0.8	(12, 13)
Tanner, 2005	CISH[1]	CISH[1]	Ni	(11)
O'Malley, 2007	2.0	2.0	0.8	(7)

Ni: Not investigated (requires FISH) ND: Not defined
[1] CISH (chromogen in situ hybridization) without CEN 17 does not allow detection of deletions

Limitations

General limitations

1. FISH is a multi-step process that requires specialized training in the selection of the appropriate reagents, as well as in tissue selection, fixation, and processing, preparation of the FISH slide, and interpretation of the staining results.
2. FISH results are dependent on the handling and processing of the tissue prior to staining. Improper fixation, washing, drying, heating, sectioning, or contamination with other tissues or fluids may influence on probe hybridization. Inconsistent results may be due to variations in fixation and embedding methods, or to inherent irregularities within the tissue.
3. For optimal and reproducible results, the tissue slides must be deparaffinized completely. The paraffin removal needs to be completed at the beginning of the staining process. (See INSTRUCTIONS FOR USE, section B.2).
4. Only temperature-calibrated water bath, heating block, and hybridization oven should be used. Use of other types of equipment may result in evaporation of *TOP2A*/CEN-17 Probe Mix during hybridization and must be validated by user.
5. The clinical significance of the presence of cancer cells with chromosome 17 polysomy has not been determined in the scientific literature. The impact of chromosome 17 polysomy on the performance of the *TOP2A* assay has not been formally assessed and the analysis of chromosome 17 polysomy has not been included in the data presented below.
6. Not all the analytical performance characteristics presented in this package insert are determined using the recommended scoring method. However, the external studies on patient samples e.g. the assay portability, pilot study and pivotal study was performed using the recommended scoring method.

Performance Characteristics

Hybridization efficiency

Hybridization efficacy of the *TOP2A* FISH pharmDx™ Kit was investigated at a routine pathology laboratory. A total of 126 formalin-fixed, paraffin-embedded tissue sections were tested using the recommended procedure. Out of the 126 specimens, 124 could be scored according to the product guidelines, while 2 specimens could not be scored owing to technical reasons. Thus, the hybridization efficacy was 124/126 = 98% (2).

FISH Analytical sensitivity

The sensitivity of the *TOP2A*/CEN-17 Probe Mix was investigated using one *TOP2A* deleted, one *TOP2A* normal and one *TOP2A* borderline-amplified cell line. The ratio between the number of *TOP2A* signals and CEN-17 signals was calculated based on a counting of 60 nuclei per cell line.

The deleted cell line was scored as deleted with an average ratio of 0.31; the normal cell line was scored as normal with an average of 1.02 while the borderline-amplified cell line was scored as borderline-amplified with an average ratio of 1.99.

Furthermore, the *TOP2A*/CEN-17 ratios of 5 *TOP2A* gene non-amplified tissue sections were determined with the *TOP2A* FISH pharmDx™ Kit. Each tissue section was scored by 3 independent technicians scoring the signals of 60 cells. Results are presented in Table 2.

Table 2. TOP2A/CEN-17 ratios in 5 non-amplified tissues scored by 3 independent technicians

	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5
Technician 1	0.95	0.94	0.92	1.03	0.99
Technician 2	1.05	0.94	0.97	1.01	1.04
Technician 3	1.03	0.99	0.96	1.05	1.04
Mean ratio	1.01	0.96	0.95	1.03	1.02
CV%	5	3	3	2	3
N	3	3	3	3	3

CV: Coefficient of variation N: Number of slides

The study confirmed all 5 tissue sections to be non-amplified with a mean *TOP2A*/CEN-17 ratio close to 1.0.

Analytical specificity

The *TOP2A* DNA probes in the *TOP2A*/CEN-17 Probe Mix have been end-sequenced and mapped to confirm a total coverage of 228 kb including the *TOP2A* gene.

The CEN-17 PNA probes in the *TOP2A*/CEN-17 Probe Mix have been tested individually and in combination to confirm their specific hybridization to the centromeric region of chromosome 17.

To exclude cross-hybridization to chromosomes other than chromosome 17, studies were performed on metaphase spreads according to standard Dako QC procedures. A total of 250 metaphase spreads were evaluated for specific hybridization of the *TOP2A* DNA and CEN-17 PNA probe mixes. In all 250 cases the hybridization was specific for chromosome 17. No cross-hybridization to loci on other chromosomes was observed in any of the 250 cases.

Robustness studies

The robustness of the *TOP2A* FISH pharmDx™ Kit was tested by varying pre-treatment time and temperature, pepsin incubation time, denaturation temperature, hybridization time and temperature, and stringent wash time and temperature.

No significant difference in results was observed at the following experimental conditions:

- Pretreatment at 7, 10 and 13 minutes combined with each of the temperatures 92 and 95-99 °C (89 °C was also tested, but resulted in weaker signals in some of the tested sections).
- Pepsin incubation times of 2, 5, 10, 15 and 18 minutes.
- Denaturation temperatures of 72, 82 and 92 °C.
- Hybridization time of 17 hours combined with each of the temperatures 40, 45 and 50 °C.
- Hybridization times of 10, 12, 14, 17 and 20 hours at a temperature of 45 °C.

The stringent wash was tested for 10 minutes at 60, 63, 65, 67 and 70 °C. Additionally, the stringent wash was tested for 5, 10 and 15 minutes at 65 °C. Stringent wash for 10 minutes at 70 °C resulted in loss of signals, whereas no significant difference in results was observed at the other time/temperature combinations. Furthermore, the following dilutions of Stringent Wash Buffer were tested: 1:10, 1:15, 1:20, 1:30 and 1:40. The 1:40 dilution of Stringent Wash Buffer resulted in loss of signals, whereas no significant difference in signal intensity was observed at the other dilutions.

Similar results were observed when the same dilutions of the Pre-Treatment Solution were tested. Again the 1:40 dilution of Pre-Treatment Solution resulted in loss of signals, whereas no significant difference in signal intensity was observed at the other dilutions.

Repeatability

The repeatability of the *TOP2A*/CEN-17 ratio was investigated with the *TOP2A* FISH pharmDx™ Kit using consecutive sections of one normal breast tissue and one breast carcinoma. The coefficient of variation was found to be 5% for both normal breast and breast carcinoma.

A total of 10 consecutive sections of breast cancer tissue with different thickness (duplicates of 3, 4, 5, 6, and 7 µm) were tested with the *TOP2A* FISH pharmDx™ Kit scoring 30 nuclei. The *TOP2A*/CEN-17 ratios were in the range of 1.02-1.10 with a mean coefficient of variation of 3%. This data support the use of sections from 4-6 microns in thickness with the *TOP2A* FISH test.

Reproducibility and Interobserver variation

The *TOP2A* FISH pharmDx™ Kit was tested for day-to-day and observer-to-observer variability using 4 different formalin-fixed and paraffin-embedded cell lines (the non-amplified MDA-231 and MDA-175, the borderline-amplified SKBR3 and the deleted MDA-361). The cell line blocks were cut into 5 µm thick sections, placed on glass slides and treated according to the standard staining protocol for tissue sections. Sections were evaluated by counting 30 nuclei per specimen. The use of cut sections from a cell line block allows for a genetically identical composition across numerous independent slides, thus eliminating the risk of genetic variation that could be seen if a similar study was conducted on tissue sections. For the day-to-day reproducibility study, a set of 5 slides for each control cell line was stained and scored on each of the four independent days. For the observer-to-observer study, a set of 15 slides was stained in the same run for each control cell line and split between the three independent observers (N=5 for each observer). The greatest *TOP2A*/CEN-17 ratio variation (10%) was found in the observer-to-observer study on the borderline-amplified cell line. This might be expected and possibly reflects certain subjectivity in signal interpretation and enumeration. Results expressed as mean ratio, standard deviation, and coefficients of variation are presented in Tables 3-4.

Table 3. Day-to-day reproducibility. *TOP2A*/CEN-17 ratio measured on 4 different days

Cell line	*TOP2A*/CEN-17 ratio	Day 1	Day 2	Day 3	Day 4	Total
MDA-231	Mean	1.04	1.03	1.03	1.02	1.03
	SD	0.04	0.05	0.02	0.02	0.03
	CV%	4	5	2	2	3
	N	5	5	5	5	20
MDA-175	Mean	1.24	1.26	1.18	1.19	1.22
	SD	0.06	0.09	0.03	0.05	0.06
	CV%	5	7	2	4	5
	N	5	5	5	5	20
SKBR3	Mean	2.01	1.94	2.08	2.00	2.01
	SD	0.17	0.14	0.14	0.08	0.14
	CV%	9	7	7	4	7
	N	5	5	5	5	20
MDA-361	Mean	0.33	0.32	0.34	0.33	0.33
	SD	0.01	0.02	0.00	0.01	0.01
	CV%	2	6	1	3	4
	N	5	5	5	5	20

SD: Standard deviation CV: Coefficient of variation N: Number of slides

Table 4. Observer-to-observer reproducibility. *TOP2A*/CEN-17 ratio measured by 3 different observers

Cell line	*TOP2A*/CEN-17 ratio	Obs. 1	Obs. 2	Obs. 3	Total
MDA-231	Mean	1.03	1.05	1.08	1.05
	SD	0.02	0.05	0.05	0.04
	CV%	2	5	5	4
	N	5	5	5	15
MDA-175	Mean	1.23	1.18	1.11	1.18
	SD	0.08	0.12	0.05	0.09
	CV%	7	10	5	8
	N	5	5	5	15
SKBR3	Mean	1.92	1.63	1.67	1.74
	SD	0.19	0.09	0.06	0.18
	CV%	10	5	4	10
	N	5	5	5	15
MDA-361	Mean	0.31	0.34	0.36	0.34
	SD	0.01	0.01	0.03	0.03
	CV%	4	3	8	8
	N	5	5	5	15

SD: Standard Deviation CV: Coefficient of Variation N. Number of slides

A second interobserver study was conducted on archived breast cancer tissue specimens selected to reflect a range of *TOP2A* ratios. Three observers counted events in 20 nuclei for each of 26 specimens (6 deleted, 16 normal and 4 amplified). Concordance between 2 out of 3 observers with regard to deletion/normal/amplification status (see Interpretation of Staining) was 100% and 96% between the third observer and the two concordant observers (1 observer scored a sample to have a ratio of 2.00, while this sample was found to be normal by the 2 other observers).

Interlaboratory reproducibility

To assess interlaboratory reproducibility (assay portability) a three-center, blinded, randomized, comparative study using formalin-fixed, paraffin-embedded (FFPE) human breast carcinoma specimens with different levels of *TOP2A* gene status (deletion, normal and amplification) was conducted.

The study involved 3 sites. Each site stained and interpreted 6 FFPE specimens in 3 separate runs. Each specimen was stained and scored 5 times at each site (total of 30 slides). A provided control was included in each run.

Table 5. Study Design and Comparison

Comparison	Procedure
Intra-assay (Within procedure reproducibility)	18 slides from 6 specimens + 1 control slide
Inter-assay (Compare staining from two previous staining runs)	6 slides + 1 control slide
Inter–technician (Compare staining results between technicians)	6 slides + 1 control slide
Inter-laboratories (Pair-wise comparisons of staining results between laboratories)	Comparison of Day 1 results between pairs of laboratories
Inter-counting methods (Compare results between 2 counting methods)	Comparison of ratio obtained by the 2 counting metods on all 90 slides

Results

The *TOP2A/CEN-17* ratio for each tested slide was reported by randomization number and by site using counts from 60 nuclei per slide. Each site designated 1 primary technician, 1 secondary technician and 1 pathologist for the staining and evaluation, respectively. Day and technician were specified for each of the specimens, and used in the statistical analysis for between day and technician evaluations.

On the first day of staining, a total of 54 slides were stained. Three replicates of each tissue were stained at each laboratory. Results are reported as *TOP2A*/CEN-17 ratio as determined when counting signals in 60 nuclei. The distribution of the ratios is presented in Table 6.

Table 6. Reported ratios obtained on the first day of staining

		***TOP2A*/CEN-17 ratio (specimens with this score)**		
Site	**Total**	**<0.80**	**0.80-2.0**	**≥ 2.00**
1	18	3	9	6
2	18	2	10	6
3	18	3	9	6
Sum	54	8	28	18

Table 7: Ratios reported for all specimens

		Overall ratio (count of specimens with this score)		
Site	**Total**	**<0.80**	**0.80-2.0**	**≥ 2.00**
1	30	5	15	10
2	30	6	14	10
3	30	5	15	10
Sum	90	16	44	30

Table 8. Reproducibility of individual tissues by two counting methods

Tissue	A1		A2		N1		N2		D1		D2	
	60 cells	60 counts	60 cells	60 counts	60 cells	60 counts	60 cells	60 counts	60 cells	60 counts	60 cells	60 counts
Mean	3.33	3.07	7.46	7.95	1.34	1.39	1.31	1.33	0.73	0.72	0.90	0.92
SD	0.73	0.73	1.84	2.31	0.14	0.20	0.12	0.15	0.06	0.07	0.14	0.16
%CV	22	24	25	29	10	15	9	11	9	10	15	17
N	15	15	10	15	15	15	15	15	15	15	15	15

SD: Standard Deviation CV: Coefficient of Variation N. Number of slides

Reproducibility was substantial for all assay comparisons across the 3 sites. There were 2 evaluations of specimen D1 with deletion status that indicated normal gene copy status and 3 evaluations of specimen D2 with an equivocal status that indicated a deleted status (Table 8). Across the 90 tests performed (Table 7), there were differences in the ratio for these 2 tissues. 13 out of 15 analyses were concordant for tissue D1 with a deletion and 12 out of 15 analyses were concordant for tissue D2 with a ratio in the equivocal zone 0.7-0.9. Gene copy status remained concordant across all tests for the other 4 tissues (2 normal and 2 amplified tissues). Evaluation of amplified versus non-amplified status across all specimens had full agreement across all study sites and all specimens.

When the alternative counting method (see Interpretation of Staining) was used, there was very high concordance between the 2 counting methods. Across all 3 sites, 89 of 90 scorings were concordant. The overall statistics including intra-assay, inter-assay and inter-observer across sites for all slides using the two counting methods is shown in Table 8.

Clinical utility

The TOP2A FISH pharmDx™ is intended to provide the physician with information about the TOP2A gene status that can be used to assess a patient's prognosis. The TOP2A gene status should be used in conjunction with other available clinical information on the patient.

Pilot study

The clinical performance of *TOP2A* FISH pharmDx™ Kit has been evaluated in a pilot study on specimens from 120 breast cancer patients (2) in collaboration with the Danish Breast Cancer Cooperative Group (DBCG). A total of 20 tumors had *TOP2A* copy number changes, almost equally divided between amplifications (n=11) and deletions (n=9). The *TOP2A* changes were not exclusively found in HER2 positive tumors, as 4 tumors (20% of the *TOP2A* abnormal cases) were HER2 negative.

The pilot study showed that 2 different counting methods gave identical results: Either the signals were counted in 60 nuclei or a total of 60 red signals were counted along with the green signals in the same nuclei. The latter method has the advantage, that the highest number of cells will be counted in the deleted and normal cases, while the lowest number of cells will be counted in the amplified cases. Amplified cases are often obvious to identify just by looking in the microscope, but are more time demanding to evaluate if 60 nuclei should be scored.

Pivotal study - DBCG 89D/*TOP2A*

In a larger scale, also conducted in Europe, the clinical performance of *TOP2A* FISH pharmDx™ Kit was evaluated based on tumor samples prospectively collected (1, 3, 17) from DBCG 89D adjuvant study (46).

Study design

The DBCG 89D study was designed as an open, prospective, randomized study. Following surgery, 980 pre- and postmenopausal women with high-risk invasive breast cancer were randomized to CMF or CEF (cyclophosphamide/epirubicin/5-fluorouracil). The primary efficacy outcome was RFS (recurrence free survival) with OS (overall survival) as a secondary endpoint. For the biological sub-

study DBCG 89D/*TOP2A* tissue blocks from the patients who had participated in the DBCG 89D study were collected from all 21 study sites and centrally analyzed retrospectively for *TOP2A* and *HER2* gene aberrations (Dako *HER2* FISH pharmDx™ Kit) as well as HER2 overexpression (Dako HercepTest™). Tissue blocks were available from 806 of the 962 patients included in the DBCG 89D study. The distribution of available samples across study sites is shown in the Table of Appendix 4.

The issue of selection bias relative to patients participating in the randomized trial is addressed, comparing the 767 patients included in the multivariate analyses with the patients not included due to unavailability of tumor tissue (n=156), unsuccessfulness of *TOP2A* test (n=33) or unknown covariates (n=6). The hypothesis of no difference in baseline values between the groups was investigated using contingency tables and χ^2–tests. Bias was detected between the two groups with respect to, tumor size, grade, menopausal status and HER2 status. The proportion of patients included in the analyses increases with the size of the tumor, and the grade, and is higher among postmenopausal women and HER2-negative women. Age, positive lymph nodes, *TOP2A* status, hormone receptor status, treatment, death, the number of events, RFS and OS showed no significant difference. Data analysis is seen in the Table of Appendix 5.

With respect to *TOP2A* and *HER2* gene aberrations the ratio was calculated as the number of signals for the gene probes divided by the number of signals for the centromere 17. Cases were scored as *HER2* or *TOP2A* FISH amplified when the ratio was ≥ 2. A *TOP2A* deletion was considered present when the ratio was < 0.8.

For the HercepTest™ all 1+, 2+ and 3+ positive specimens were subject to *HER2* FISH analysis. The scoring of HER2 positivity (47) in the study was as following: Positive: HercepTest=3+, or HercepTest=2+ and FISH *HER2* ratio ≥ 2.0. Negative: HercepTest=0, 1+, or HercepTest=2+ and FISH *HER2* ratio < 2.0

The clinical study (DBCG 89D) (46) and the biological sub-study (DBCG 89D/*TOP2A*) (1, 3) were conducted according to the Helsinki declaration and approved by the local Ethical Committees.

Statistical methodology

The primary endpoint for the study was RFS, defined as the time from randomization to an event or censoring. An event was defined as relapse local or distant, second malignancy or death, whichever comes first. Censoring was due to 'lost to follow up', 'patient will no longer participate' or 'alive without disease at end of follow-up'.

The secondary end point was OS, defined as the time from randomization to death (irrespective of the cause) or censoring. Patients were considered as censored if patients were alive at end of follow-up.

The effect of *TOP2A* status within treatment groups is illustrated by figures of the univariate survival curves.

The Cox proportional hazards model was adjusted according to the results of the goodness-of-fit procedures, defining the basic multivariate Cox model for analysis of RFS and OS. The hazards ratio (HR), the 95 % confidence interval and the p-value of the Wald test was given for each covariate in the Cox model.

Correlations between *TOP2A* status and clinical and pathological variables including HER2-status were tested by χ2-test.
Follow-up time was quantified in terms of a Kaplan-Meier estimate of potential follow-up. Analyses were performed for possible selection bias using the χ2-test and log-rank test.

Patients with missing clinical covariates (N=6), except for receptor status, were excluded from the multivariate analyses in the Cox proportional hazard model.

Results

The *TOP2A* FISH analysis was successful on 773 of the 806 (96%) breast cancer samples. The overall distribution of *TOP2A* status among the eligible patients is shown in Table 9. Amplification of *TOP2A* was seen in 92 (11.9%) of the 773 eligible patients and deletion in 87 (11.3%).

Table 9. Distribution of *TOP2A* status

TOP2A status	N (%)
Deleted	87 (11.3)
Normal	594 (76.8)
Amplified	92 (11.9)
Total	773 (100)

The distribution of *TOP2A* amplifications and deletions in relation to the HER2 status is shown in Table 10.

Table 10. Distribution of HER2 status in relation to *TOP2A* status

	TOP2A	N N (%)	Deleted N (%)	Normal N (%)	Amplified N (%)	p-value χ^2
HER2 Status	Negative	527 (100)	26 (4.9)	487 (92.4)	14 (2.7)	p< 0.0001
	Positive	246 (100)	61 (24.8)	107 (43.5)	78 (31.7)	
	Total	773 (100)	87 (11.3)	594 (76.8)	92 (11.9)	

HER2 status was defined as positive if HercepTest=3+ or HercepTest=2+ and FISH *HER2* ratio ≥ 2.0 and negative if HercepTest=0, 1+, or HercepTest=2+ and FISH *HER2* ratio < 2.0

Looking at the HER2 status, more *TOP2A* aberrations were found among the HER2 positive tumors. *TOP2A* aberrations were seen in 139 (56.5%) of the 246 HER2 positive tumors and in 40 (7.6%) of the 527 HER2 negative tumors. Thus, 40 (22.3%) patients with *TOP2A* aberrations would have been overlooked, if only the HER2 positive samples had been analyzed.

The distribution of *TOP2A* aberrations in relation to the *HER2* FISH status is shown in Table 11. As for HER2 status a significant correlation with *TOP2A* status was found.

Table 11. Distribution *HER2* FISH in relation to *TOP2A* FISH

	TOP2A	N N (%)	Deleted N (%)	Normal N (%)	Amplified N (%)	p-value χ^2
***HER2* FISH**	Normal	539 (100)	31 (5.8)	493 (91.5)	15 (2.8)	p< 0.0001
	Amplified	234 (100)	56 (23.9)	101 (43.2)	77(32.9)	
	Total	773 (100)	87 (11.3)	594 (76.8)	92 (11.9)	

HER2 FISH ratio ≥ 2.0 is defined as amplified and normal if *HER2* FISH ratio < 2.0

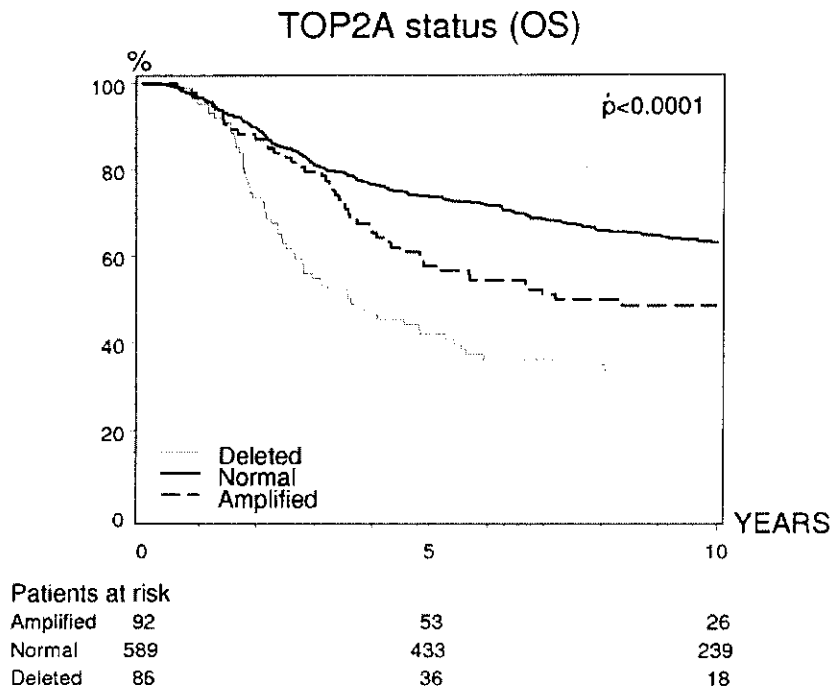
The distribution of *TOP2A* aberrations in relation to the HercepTest™ score is shown in Table 12. As for HER2 status a significant correlation with *TOP2A* status was found.

Table 12. Distribution of HercepTest™ score in relation to *TOP2A* status

	TOP2A	N N (%)	Deleted N (%)	Normal N (%)	Amplified N (%)	p-value χ^2
HercepTest	0	209 (100)	11 (5.3)	192 (91.9)	6 (2.9)	p< 0.0001
	1+	257 (100)	13 (5.1)	238 (92.6)	6 (2.3)	
	2+	78 (100)	3 (3.9)	65 (83.3)	10 (12.8)	
	3+	229 (100)	60 (26.2)	99 (43.3)	70 (30.6)	
	Total	773 (100)	87 (11.3)	594 (76.8)	92 (11.9)	

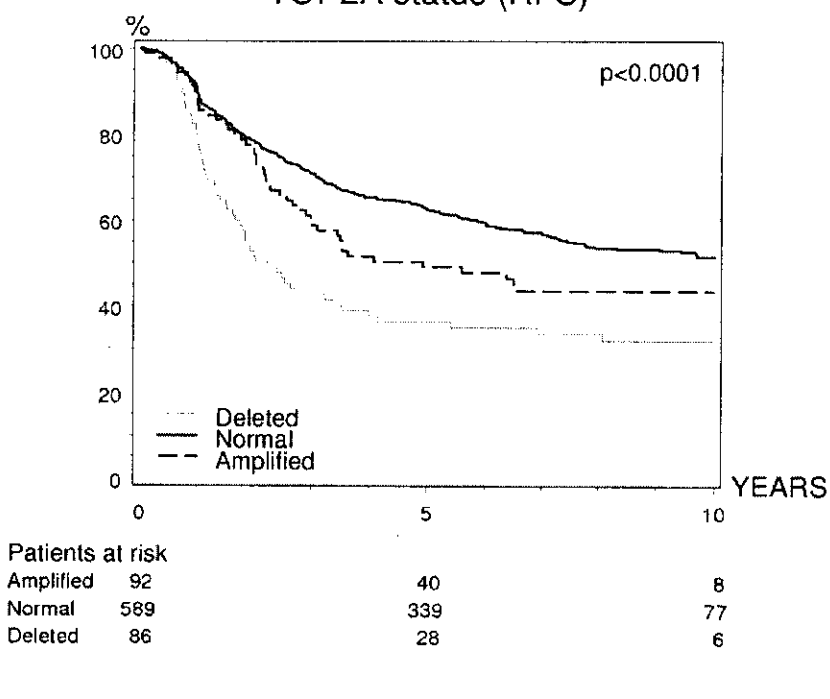
Six of the 773 patients with *TOP2A* data available had missing clinical data that excluded them from the multivariate analyses.

An analysis of the distribution of the *TOP2A* aberrations (amplifications and deletions) with respect to the baseline characteristics showed a significant association with several of the established histopathological prognostic factors. Further, the data demonstrated that the proportion of women with *TOP2A* aberrations was increasing with age resulting in a higher frequency among postmenopausal than premenopausal women. The univariate survival analyses indicated a negative significant effect on both RFS and OS, as patients with amplifications and deletions had a significant reduction in survival compared to patients with a normal *TOP2A* status. The survival curves also indicated that patients with deletions had an even worse prognosis than patients with an amplified or normal *TOP2A* status. The survival curves for patients with amplified, normal or deleted *TOP2A* status are shown in Figure 1.



Patients at risk

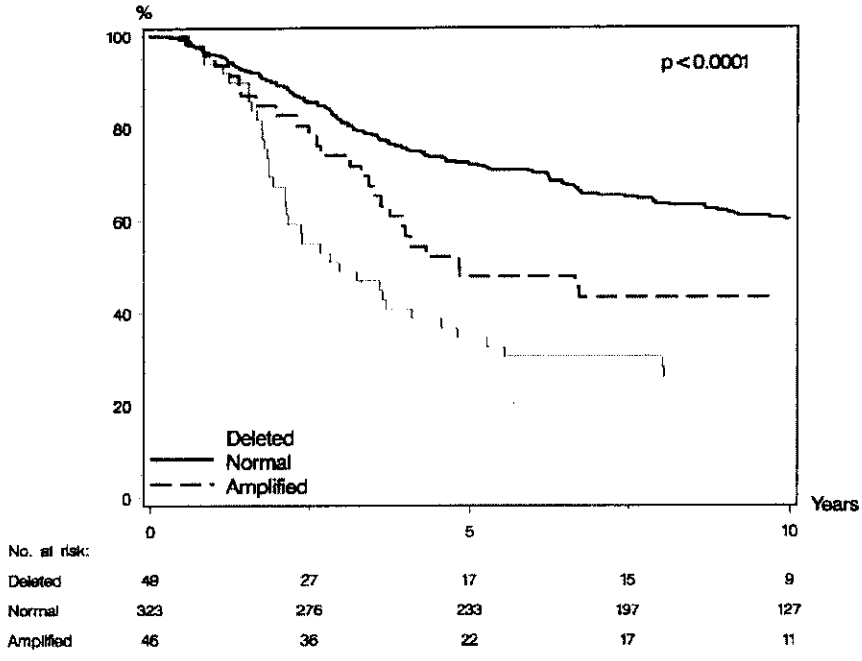
Amplified	92	53	26
Normal	589	433	239
Deleted	86	36	18



Patients at risk

Amplified	92	40	8
Normal	589	339	77
Deleted	86	28	6

Figure 1. Overall survival (OS, upper panel) and Recurrence free survival (RFS, lower panel) for patients in the 3 *TOP2A* groups (normal, amplified and deleted *TOP2A* status) showing a significant worse outcome for patients with *TOP2A* amplification and even poorer for patients with *TOP2A* deletion. Results were combined across treatment arms.



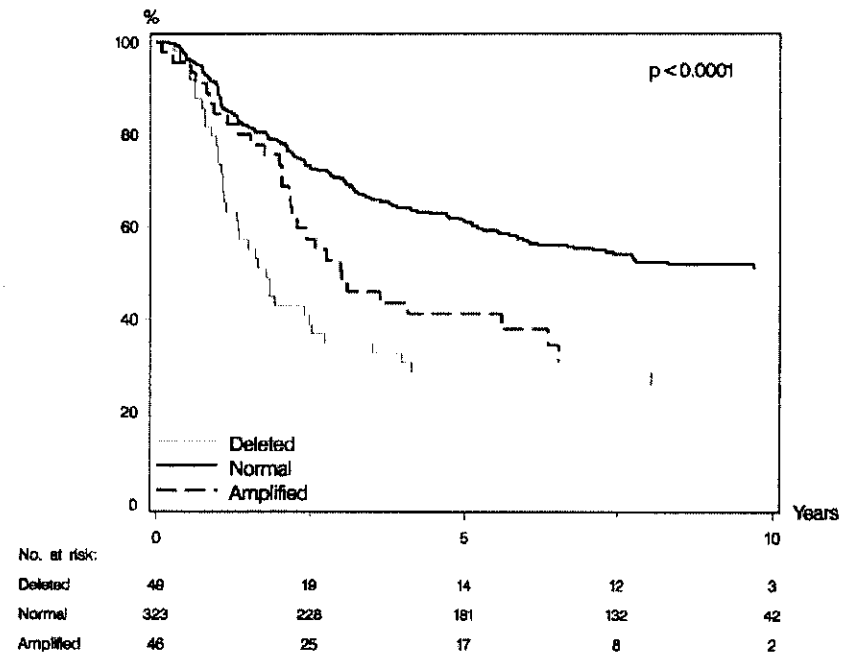
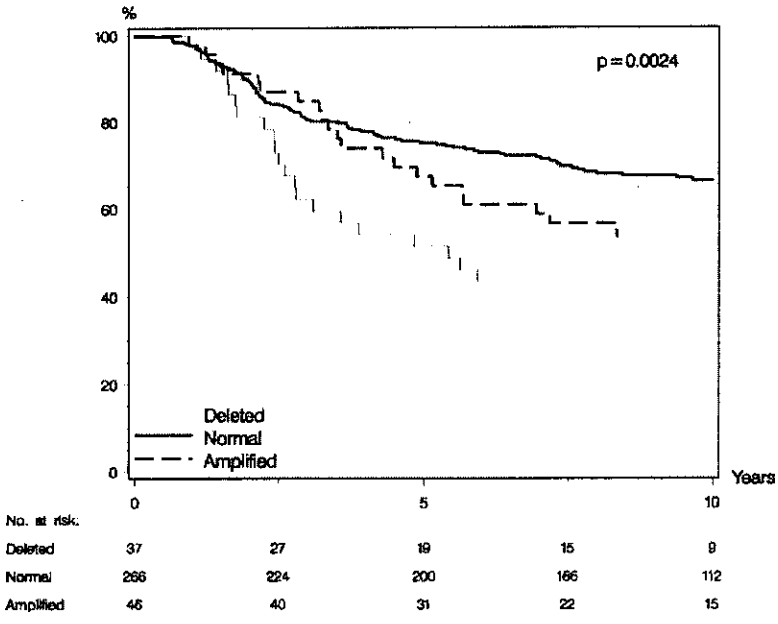


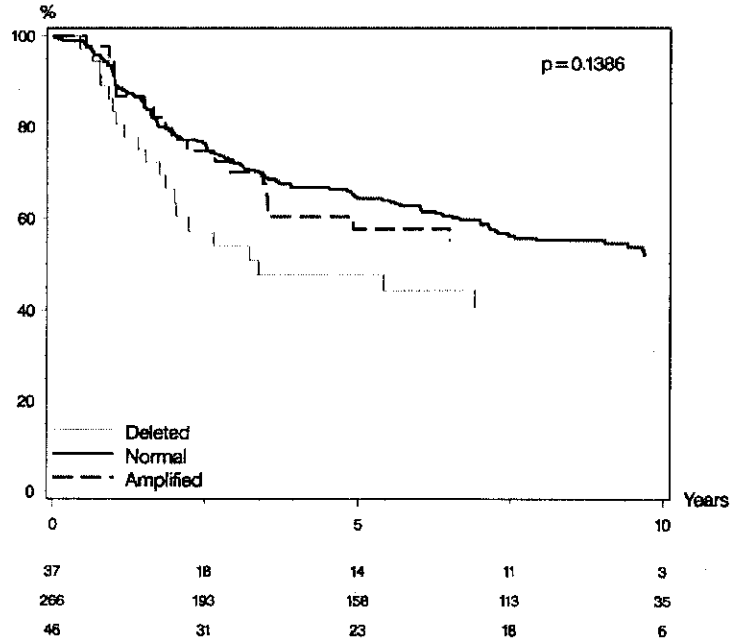
Figure 2. Overall survival (OS, upper panel) and Recurrence free survival (RFS, lower panel) for patients treated with CMF. Patients with normal *TOP2A* status have a significant better outcome than patients with *TOP2A* amplification or deletion.

OS: CEF by TOP2A status



No. at risk:					
Deleted	37	27	19	15	9
Normal	266	224	200	166	112
Amplified	46	40	31	22	15

RFS: CEF by TOP2A status



No. at risk:					
Deleted	37	18	14	11	3
Normal	266	193	158	113	35
Amplified	46	31	23	18	6

Figure 3. Overall survival (OS, upper panel) and Recurrence free survival (RFS, lower panel) for patients treated with CEF. For OS patients with *TOP2A* amplifications and deletions had a significant worse outcome than patients with *TOP2A* normal status. For RFS, patients with *TOP2A* amplification had outcome comparable to patients with *TOP2A* normal status.

Besides the association with the established clinical prognostic factors it was shown that *TOP2A* aberrations had an independent prognostic value. Using the Cox proportional hazard model it was demonstrated that a *TOP2A* gene aberration was associated with a significant worse prognosis in the CMF treatment arm both with respect to RFS (P=0.0209 and OS (P=0.0102). The HR (Hazard Ratio) and the 95% confidence limits based on Cox model for RFS and OS are shown in Table 13 and 14.

The basic multivariate Cox model included the prognostic parameters: menopausal status, tumor size, number of positive lymph nodes, HER2 and *TOP2A* status, malignancy grade and receptor status. The prognostic value of positive lymph node status and *TOP2A* varied within treatment arms and thus separate coefficients are provided for each. The poor prognosis associated with deleted or amplified status within the CMF arm remains statistically significant after considering other clinical variables but is no longer significant in the CEF arm. This was consistent for both RFS and OS.

Table 13. Hazard Ratio (HR) for Recurrence Free Survival (RFS)

Variable	P-value	HR	95% CI
Menopause	0.0673		
Pre		1	
Post		1.25	(0.98-1.59)
Tumour size pr. increasing cm	<0.0001	1.15	(1.09-1.22)
Treatment	0.4173		
CMF		1	
CEF		0.82	(0.51-1.32)
Positive lymph nodes within treatment CEF :	<0.0001		
0		1	
1-3		2.56	(1.53-4.28)
4-		4.21	(2.56-6.92)
Positive lymph nodes within treatment CMF :	<0.0001		
0		1	
1-3		1.71	(1.09-2.67)
4-		4.16	(2.69-6.43)
TOP2A status within treatment CEF :	0.1923		
Deleted		1.07	(0.64-1.77)
Normal		1	
Amplified		0.64	(0.38-1.08)
TOP2A status within treatment CMF	0.0209		
Deleted		1.63	(1.10-2.42)
Normal		1	
Amplified		1.55	(1.00-2.41)
HER2 status	0.2309		
Negative		1	
Positive		1.17	(0.90 -1.53)

Table 14. Hazard Ratio (HR) for Overall Survival (OS)

Variable	P-value	HR	95% CI
Menopause	0.0135		
Pre		1	
Post		1.36	(1.07-1.75)
Tumour size	<0.0001		
pr. increasing cm		1.17	(1.10-1.24)
Treatment	0.0059		
CMF		1	
CEF		0.39	(0.20-0.76)
Positive lymph nodes within treatment CEF	<0.0001		
0		1	
1-3		5.51	(2.76- 11.02)
4-		9.77	(4.96-19.25)
Positive lymph nodes within treatment CMF	<0.0001		
0		1	
1-3		1.74	(1.06-2.85)
4-		4.09	(2.53-6.62)
TOP2A status within treatment CEF	0.1305		
Deleted		1.28	(0.78-2.10)
Normal		1	
Amplified		0.70	(0.42-1.16)
TOP2A status within treatment CMF	0.0102		
Deleted		1.84	(1.23-2.75)
Normal		1	
Amplified		1.40	(0.89-2.21)
HER2 status	0.0414		
Negative		1	
Positive		1.33	(1.01-1.75)

The estimates of the effect due to *TOP2A* status are similar with respect to OS as to those found in the analysis of RFS.

The prognostic value of the HER2 status was also investigated and the univariate survival analyses indicated a significant negative effect on both RFS and OS, as HER2 positive patients had a reduction in survival compared to patients with a normal HER2 status. The primary analysis of RFS using the Cox proportional hazard regression analysis showed no significant effect of the HER2 status. When repeating the analysis with respect to OS the positive HER2 status came out having a significant negative impact on survival.

The effect of the interaction between *TOP2A* status and HER2 status investigated by using a multivariate Cox-models in HER2-positive patients only, showed significant effect of *TOP2A* status (P=0.011) for RFS and (P=0.048) for OS. However, because of the limited number of patients that were *TOP2A* amplified but HER2 negative the relationship of these two markers as prognostic factors could not be fully explored.

Discussion and Conclusion

The DBCG 89D/*TOP2A* study has demonstrated significant prognostic value of *TOP2A* gene amplifications and deletions. Based on the comparisons to HER2 status it can be concluded that the HER2 status and *TOP2A* status are not interchangeable for the prognostic value.

TOP2A is a molecular target for the pharmacological action of anthracyclines. Anthracycline-based chemotherapy with doxorubicin or epirubicin is among the most active regimens in breast cancer (25, 26). However, these compounds possess significant acute and long-term serious side effects, such as cardiotoxicity and leukemia. The presence of predictive implications from *TOP2A* amplifications for optimal use of anthracycline-containing therapy is an area of active research with promising initial results that require confirmation and extension in a context of currently available chemotherapeutic options. Whether *TOP2A* amplification is an independent predictive marker of response to (chemotherapy or immune-based) remains to be established.

Troubleshooting

Problem	Probable Cause	Suggested Action
1. No signals or weak signals	1a. Kit has been exposed to high temperatures during transport or storage	1a. Check storage conditions. Ensure that dry ice was present when the shipment was received. Ensure that vials 2, 3 and 5 have been stored at maximum 2-8 °C, and that vials 3 and 5 have been stored in the dark.
	1b. Microscope not functioning properly - Inappropriate filter set - Improper lamp - Mercury lamp too old - Dirty and/or cracked collector lenses - Unsuitable immersion oil	1b. Check the microscope and ensure that the used filters are suitable for use with the kit fluorochromes, and that the mercury lamp is correct and has not been used beyond expected lifetime. (see Appendix 3). In case of doubt, please contact your local microscope vendor.
	1c. Faded signals	1c. Avoid long microscopic examination and minimize exposure to strong light sources.
	1d. Pre-treatment conditions incorrect	1d. Ensure that the recommended pre-treatment temperature and time are used.
	1e. Evaporation of Probe Mix during hybridization	1e. Ensure sufficient humidity in the hybridization chamber
2. No green signals	2a. Stringent wash conditions incorrect	2a. Ensure that the recommended stringent wash temperature and time are used, and that coverslips are removed before performing stringent wash
3. No red signals	3a. Pre-treatment conditions incorrect	3a. Ensure that the recommended pre-treatment temperature and time are used
4. Areas without signal	4a. Probe volume too small	4a. Ensure that the probe volume is large enough to cover the area under the coverslip
	4b. Air bubbles caught during Probe Mix application or mounting	4b. Avoid air bubbles. If observed, gently tap them away using forceps
5. Excessive background staining	5a. Inappropriate tissue fixation	5a Ensure that only formalin-fixed, paraffin-embedded tissue sections are used
	5b. Paraffin incompletely removed	5b. Follow the deparaffinization and rehydration procedures outlined in Section B.2
	5c. Stringent wash temperature too low	5c. Ensure that the stringent wash temperature is 65 (±2) °C

Problem	Probable Cause	Suggested Action
	5d. Prolonged exposure of hybridized section to strong light	5d. Avoid long microscopic examination and minimize exposure to strong light
6. Poor tissue morphology	6a. Incorrect Pepsin treatment	6a. Adhere to recommended Pepsin incubation times. See section B.3, step 2. Ensure that the Pepsin is handled at the correct temperature. See Section B.1
	6b. Incorrect pre-treatment conditions may result in unclear or cloudy appearance	6b. Ensure that the recommended pre-treatment temperature and time are used
	6c. Too long Pepsin treatment or very thin section thickness may cause ghost cells or donut cells to appear.	6c. Shorten the Pepsin incubation time. See section B.3, step 2. Ensure that the section thickness is 4-6 µm.

NOTE: If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please call our Technical Services for further assistance.

Appendix 1

***TOP2A* FISH pharmDx™ Kit, Code K5333**
Protocol Checklist

Staining Run Log ID:_____

Date (Day 1) of the run: _____

TOP2A FISH pharmDx™ Kit, K5333 Lot: _____

Specimen ID: _____

Equipment ID: _____

Date of dilution/expiration of the 1x Wash Buffer (Vial 6 diluted 1:20):_____/_____

Tissue fixed in neutral buffered formalin	**Yes** □ **No** □

DAY 1

Step 1: Pre-Treatment	
Date of dilution/expiration of the Pre-Treatment Solution (Vial 1 diluted 1:20)	/
Measured temperature of Pre-Treatment Solution (95-99 °C)	°C
Pre-treatment (10 minutes), and cooling (15 minutes)	
Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes)	
Step 2: Pepsin	
Duration of Pepsin (Vial 2) treatment (5-15 minutes)	Minutes
Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes)	
Dehydrate slides (3 x 2 minutes) in graded series of ethanol and air dry	
Step 3: *TOP2A*/CEN-17 Probe Mix	
Apply Probe Mix (Vial 3), coverslip and seal with Coverslip Sealant	
Measured denaturation temperature (82 ±2 °C)	°C
Denaturation for 5 minutes	
Measured hybridization temperature (45 ±2 °C)	°C
Hybridization overnight (protect from light)	

DAY 2

Step 4: Stringent Wash	
Date of dilution/expiration of the Stringent Wash Buffer (Vial 4 diluted 1:20)	/
Measured temperature of Stringent Wash Buffer (65 ±2 °C)	°C
Stringent wash (10 minutes) after removing the coverslips	
Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes)	
Dehydrate slides (3 x 2 minutes) in graded series of ethanol and air dry	
Step 5: Mounting	
Apply 15 µL of Fluorescence Mounting Medium (Vial 5) and coverslip	

Comments:_____

_____

Date and signature, Technician: _____

Appendix 2

TOP2A FISH pharmDx™ Kit, Code K5333
Scoring Scheme

Staining Run Log ID: _____

Date (Day 1) of the run: _____

TOP2A FISH pharmDx™ Kit, K5333 Lot: _____ Specimen ID: _____

Nucleus No.	*TOP2A* score	CEN-17 score	Nucleus No.	*TOP2A* score	CEN-17 score	Nucleus No.	*TOP2A* score	CEN-17 score
1			21			41		
2			22			42		
3			23			43		
4			24			44		
5			25			45		
6			26			46		
7			27			47		
8			28			48		
9			29			49		
10			30			50		
11			31			51		
12			32			52		
13			33			53		
14			34			54		
15			35			55		
16			36			56		
17			37			57		
18			38			58		
19			39			59		
20			40			60		
Total (1-20)			Total (21-40)			Total (41-60)		

For determination of the _TOP2A_/CEN-17 ratio, count the number of _TOP2A_ signals and the number of CEN-17 signals in either 60 nuclei or the number of nuclei required for 60 _TOP2A_ signals (see Interpretation of Staining section). Divide the total number of _TOP2A_ signals by the total number of CEN-17 signals. If the _TOP2A_/CEN-17 ratio is borderline (0.7-0.9 or 1.8-2.2), recount the specimen and recalculate the ratio based on all counted cells.

Number of counted cells	*TOP2A signals*	CEN-17 signals	*TOP2A/CEN-17 ratio*

- ☐ _TOP2A_ gene deletion (Ratio < 0.8)
- ☐ _Normal TOP2A_ gene status (0.8 ≤ Ratio < 2)
- ☐ _TOP2A_ gene amplification (Ratio ≥ 2)

Date and signature, Technician: _____

Date and signature, Pathologist: _____

Comments: _____

For scoring guidelines: see Interpretation of Staining.

Appendix 3

Fluorescence Microscope Specifications

Dako recommends the following equipment for use with the *TOP2A* FISH pharmDx™ Kit, K5333:

1. Microscope type

- Epifluorescence microscope.

2. Lamp

- 100 watt mercury lamp (keep record of burning time).

3. Objectives

- For screening of the tissue, fluorescence dry 10X or fluorescence oil immersion 16X objectives are applicable.
- For high power magnification and scoring of signals, only fluorescence oil immersion objectives, e.g. 100X are recommended.

4. Filters

Filters are individually designed for specific fluorochromes and must be chosen accordingly. Dako recommends the use of a specific DAPI filter in combination with a high quality Texas Red/FITC double filter.

- DAPI filter, e.g. Chroma filter # 31000.
- Texas Red/FITC double filter, e.g. Omega Optical filter # XF53 or Chroma filter # 51006.
- Texas Red and FITC single filters can be used for confirmation.

Fluorochrome	Excitation Wavelength	Emission Wavelength
FITC	495 nm	520 nm
Texas Red	596 nm	615 nm

Filters are specific to each microscope type and the use of appropriate filters is crucial for the interpretation. If you want detailed information, please contact your microscope provider or your Dako representative.

5. Oil

- Non-fluorescing oil.

Precautions

- A 50 watt mercury lamp is not recommended.
- Rhodamine filters cannot be used.
- Triple filters are not recommended.

A non-optimized microscope may cause problems when reading the fluorescent signals. It is important that the light source has not expired and that it is properly aligned and focused.

Customers should monitor and follow the manufacturer's recommendations for the mercury lamp. The microscope should be maintained and the mercury lamp should be in alignment prior to interpreting results.

An effort should be made to expose the sample to as little of the excitation light as possible in order to minimize fading of the fluorescence.

We recommend that you discuss the set-up of your particular microscope with the manufacturer before starting the fluorescence in situ hybridization, or refer to the literature.

Appendix 4

Distribution of Patients and Samples across all 21 Study Sites, all Patients (N=980)

Distribution of number of patients and samples across study sites	Subpopulations of patients							
	No adjuvant chemo therapy		No tissue available		*TOP2A* testing unsuccessful		*TOP2A* testing successful	
	N	%	N	%	N	%	N	%
Randomization Center								
RIGSHOSPITALET	4	1.8	39	18.0	9	4.1	165	76.0
BISPEBJERG	.	.	2	6.9	2	6.9	25	86.2
HERLEV	5	3.7	30	22.1	2	1.5	99	72.8
ROSKILDE	1	1.8	4	7.3	1	1.8	49	89.1
HOLBÆK	.	.	.	.	.	.	1	100.0
SLAGELSE	.	.	1	50.0	.	.	1	50.0
NÆSTVED	1	2.1	13	27.7	2	4.3	31	66.0
NAKSKOV	1	5.0	2	10.0	.	.	17	85.0
RØNNE	.	.	.	.	.	.	10	100.0
ODENSE	3	2.8	14	13.0	.	.	91	84.3
SØNDERBORG	2	7.7	1	3.8	1	3.8	22	84.6
HADERSLEV	.	.	5	25.0	1	5.0	14	70.0
ÅBENRÅ	.	.	.	.	.	.	1	100.0
ESBJERG	.	.	17	22.7	.	.	58	77.3
VEJLE	1	2.4	5	11.9	10	23.8	26	61.9
HOLSTEBRO	.	.	2	20.0	.	.	8	80.0
HERNING	.	.	2	15.4	.	.	11	84.6
ÅRHUS KH	.	.	8	12.7	3	4.8	52	82.5
VIBORG	.	.	2	11.8	1	5.9	14	82.4
ÅLBORG	.	.	7	11.9	.	.	52	88.1
HJØRRING	.	.	2	6.9	1	3.4	26	89.7
All	18	1.8	156	15.9	33	3.4	773	78.9

Appendix 5

Distribution of Baseline Characteristic vs. Analyses Groups

		N	÷ Multivariate Analyses N (%)	+ Multivariate Analyses N (%)	p-value
All		962	195[1]	767	-
HER2 Status	HER2 negative	542	19 (50.0)	523 (68.2)	0.02[2]
	HER2 positive	263	19 (50.0)	244 (31.8)	
***TOP2A* Status**	Deletion	87	1 (16.7)	86 (11.2)	-[4]
	Normal	594	5 (83.3)	589 (76.8)	
	Amplification	92	0 (0.0)	92 (12.0)	
Events	No event	508	108 (55.4)	400 (52.2)	0.42[2]/0.32[3]
	Event	454	87 (44.6)	367 (47.9)	
Death	Alive	546	112 (57.4)	434 (56.6)	0.83[2]/0.4[3]
	Dead	416	83 (42.6)	333 (43.4)	
Treatment	CEF	447	98 (50.3)	349 (45.5)	0.232[2]
	CMF	515	97 (49.7)	418 (54.5)	
Menopause	Pre	688	156 (80.0)	532 (69.4)	0.0033[2]
	Post	274	39 (20.0)	235 (30.6)	
Age at surgery (yrs.)	-39	163	39 (20.0)	124 (16.2)	0.075[2]
	40-49	472	105 (53.9)	357 (47.9)	
	50-59	201	31 (15.9)	170 (22.2)	
	60-69	126	20 (16.7)	106 (13.8)	
Tumor size (mm)	0-20	422	107 (56.6)	315 (41.1))	< 0.001[2]
	21-50	461	72 (38.1)	389 (50.7)	
	-51	73	10 (5.3)	63 (8.2)	
No. of positive nodes	None	352	70 (35.9)	282 (36,8)	0.075[2]
	1-3	321	77 (39.5)	244 (31.8)	
	-4	289	48 (24.6)	241 (31.4)	
Malignancy grade	I	128	29 (15.7)	99 (12.9)	0.002[2]
	II	468	108 (58.4)	360 (47.0)	
	III	356	48 (26.0)	308 (40.2)	
Receptor status	Positive/Unknown	250	48 (24.6)	202 (26.3)	0.62[2]
	Negative	712	147 (75.4)	565 (73.7)	

[1] 195 patients were not included in the multivariate analyses due to unavailability of tumor tissue (n=156), unsuccessfulness of *TOP2A* test (n=33) or unknown covariates (n=6).

[2] χ^2-test

[3] Log-rank test in univariate survival analysis

[4] No test performed due to the small number, however no bias observed in the estimated frequencies,

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Explanation of symbols

REF	Catalogue number	-20°C 8°C	Temperature limitation	LOT	Batch code		Toxic
IVD	In vitro diagnostic medical device		Keep away from sunlight (consult storage section)		Use by	F+	Extremely flammable
	Consult instructions for use	Σ	Contains sufficient for <n> tests		Manufacturer	N	Dangerous for the environment

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